Dkt. 0575/55424-A-PCT-US/JPW/AJM/JCS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Ann Marie Schmidt, et al.

U.S. Serial No.: 09/689,469 Examiner: C. Yaen

Filed : October 12, 2000 Group Art Unit: 1643

For : A METHOD FOR INHIBITING TUMOR INVASION OR SPREADING IN A SUBJECT

1185 Avenue of the Americas
New York, New York 10036

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SIR:

DECLARATION UNDER 37 C.F.R. §1.132

I, Ann Marie Schmidt, M.D., hereby declare that:

1. I am a co-inventor named in the above-identified patent application.

2. I am a professor of surgical sciences at Columbia University in New York, New York. A copy of my *curriculum vitae* is attached hereto as **Exhibit A**.

3. I have reviewed and am familiar with pending claims 57-60 and 76-78 of the subject application. I understand that pending claims 57-60 and 76-78 provide a method for identifying an agent which inhibits tumor invasion in a local cellular environment. I also understand that this

method comprises: (a) providing a solid support coated with amphoterin; (b) contacting the solid support with a tumor cell which expresses receptor for advanced glycation endproducts (RAGE) under appropriate cell culture conditions for cell migration and growth; (c) admixing to the tumor cell culture of step (b) an agent to be tested; (d) determining the amount of spreading of the tumor cells on the solid support; and (e) comparing the amount of spreading of the tumor cells determined in step (d) with the amount of spreading determined in an identical tumor cell culture in the absence of the agent, wherein a decrease in the amount of spreading determined in step (d) indicates that the agent is identified as an agent which inhibits tumor invasion in the local cellular environment.

4. I have read and am familiar with the July 12, 2005 Advisory Action issued by the U.S. Patent and Trademark Office in connection with the subject application. I also understand that in the Advisory Action, the Examiner has maintained the rejection of claims 57-60 and 76-78 as allegedly obvious under 35 U.S.C. §103, which rejection was made in the January 26, 2005 Final Office Action issued by the U.S. Patent and Trademark Office. I have also read and am familiar with the January 26, 2005 Final Office Action and the references of Hori, et al. (J. Biol. Chem. 1995; 270(43):25752-25761) ("Hori"), Miki, et al. (Biochem. Biophys. Res. Commun. 1993 Oct. 29:196(2):984-9) ("Miki") and Parkkinen, et al. (J. Bio.Chem. 1993 Sept. 268(26):19726-19738) ("Parkkinen") cited by the Examiner

in support of this rejection.

5. In the January 26, 2005 Final Office Action, I understand the Examiner to assert that the cited references, when taken together, create, among other things, a motive to identify agents that inhibit tumor invasion by determining whether the agents disrupt the interaction between RAGE and amphoterin.

6. I am a co-author of the article entitled "Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases" (Taguchi, et al., Nature 405:354-360 (2000)) ("Taguchi"), annexed hereto as **Exhibit B**. Taguchi describes certain experimental findings incorporated into the subject application, namely, that RAGE-amphoterin interaction is a pathway for tumor invasion and that this pathway is not bypassed by a compensatory or collateral pathway.

7. I am also familiar with the article entitled "Cancer: Checkpoint for Invasion" (Liotta and Clair, Nature 405:287-288 (2000)) ("Liotta"), annexed hereto as **Exhibit C**. I understand Liotta is a review of the findings set forth in Taguchi. In the first paragraph, Liotta states, in part, that "Taguchi and colleagues. . . have now identified proteins called RAGE and amphoterin as a receptor-ligand pair in a molecular checkpoint that regulates not only the invasiveness but also the growth and movement of tumour cells - the trio of characteristics

required for malignancy." I understand this statement to mean that prior to the findings in Taguchi, it was not known that RAGE-amphoterin is a molecular checkpoint regulating tumor invasion, growth and movement.

8. In the fourth paragraph, Liotta states, in part, that "the best way to link a molecule causally to malignancy is to start with a cell that is already malignant, and to attempt to block the molecule or pathway of interest. This was the tack taken by Taguchi et al." I understand this statement to mean that prior to the findings of Taguchi, no causal link was known to exist between RAGE-amphoterin interaction and tumor invasion.

9. In the last paragraph, Liotta states, in part, that "[t]he trick is to find a rheostat in the cell's circuitry that is not bypassed by collateral or compensatory paths." I understand this statement to mean that prior to the findings set forth in Taguchi, it was not established that the RAGE-amphoterin pathway is not bypassed by a collateral or compensatory pathway.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize

the validity of the subject application or any patent issuing thereon.

amc

Ann Marie Schmidt, M.D.

10/28/05

Date

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CURRICULUM VITAE

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EDUCATION

University	Degree/Field	Year
New York University Washington Square School of the Arts & Sciences New York, New York	B.A. Summa Cum Laude Biology & History	1979
New York University School of Medicine New York, New York	M.D. with Honors	1983

AWARDS AND HONORS

Dean's List	1975-1979
Phi Beta Kappa	1978
Alpha Omega Alpha	1982
Juvenile Diabetes Foundation Fellowship	1990-1992

Harold and Golden Lamport Prize for Excellence in Clinical Research (Columbia University)	1998
American Society of Clinical Investigation	1999
Established Investigator of the American Heart Association	1999
Recipient, Burroughs Wellcome Fund Clinical Scientist Award in Translational Research	1999
Schunk- Prize for Medicine, 1999 Justus-Liebig-University Gießen, Germany	1999
Distinguished Lecturer Department of Oral Biology State University of New York at Buffalo School of Dentistry	2000
Co-director, Juvenile Diabetes Research Foundation International Center for Complications at Columbia University	2000-2002
Director, Juvenile Diabetes Research Foundation International Center for Complications at Columbia University	2002-2003
Keynote Lecturer, Banting and Best Diabetes Centre Annual Scientific Day, University of Toronto, Toronto, Canada	2002
Opponent in the Dissertation of the Degree of Doctor of Philosophy by Henri Huttunen, Dept of Biochemistry, University of Helsinki, Helsinki, Finland	2002
Mary Jane Kugel Award Juvenile Diabetes Research	2003

Foundation International

Gerald and Janet Carrus Professor of Surgical Science	2003-present
Director, Juvenile Diabetes Research Foundation International Center for Complications at Columbia University	2004-2006

SPECIALTY BOARDS Internal Medicine, American Board of Internal Medicine 1988

LICENSURE New York State Medical License Number: 159704

PROFESSIONAL MEMBERSHIPS

American Society of Hematology
American Diabetes Association
American Heart Association, Thrombosis Council
American Society of Clinical Investigation
Society for Neuroscience
American Association for Cancer Research

RESEARCH AND/OR PROFESSIONAL EXPERIENCE

Intern, Internal Medicine, New York University Medical Center, Bellevue Hospital Center, July, 1983 - June, 1984.

Resident, Internal Medicine, New York University Medical Center, Bellevue Hospital Center, July, 1984 - June, 1987.

Chief Resident, Internal Medicine, New York University Medical Center, Bellevue Hospital Center, July, 1987- June, 1988.

Fellow, Hematology, New York University Medical Center, Bellevue Hospital Center, July, 1988 - June, 1989.

Fellow, Medical Oncology, New York University Medical Center, Bellevue Hospital Center, July, 1989 - June, 1990.

Teaching Assistant, Internal Medicine, New York University School of Medicine, New York, New York, 1983-1990.

Post-Doctoral Research Fellow, Columbia University, Department of Physiology and Cellular Biophysics, Laboratory of Dr. David Stern, July, 1990 - June, 1993.

Assistant Professor, Columbia University, Department of Medicine, Division of Molecular Medicine, July, 1993 - November, 1998.

Assistant Professor, Columbia University, Department of Surgery, January 1995-November 1998.

Associate Professor, Division of Surgical Science, Department of Surgery, with tenure, December 1, 1998 - June 30, 2003.

Division Chief, Division of Surgical Science, Department of Surgery, June, 2002 - present

Professor, Division of Surgical Science, Department of Surgery, July 1, 2003 - present

Gerald and Janet Carrus Professor of Surgical Science, October, 2003-present

COMMITTEE MEMBERSHIPS, MEETING CHAIRMANSHIPS, AND PLENARY SESSIONS:

1996 Co-chairperson: Session on "Featured Research - Oxidant Signaling and Gene Regulation", American Heart Association, National Meeting, New Orleans, Louisiana

1997 Co-chairperson: Session on Diabetes and Endothelial Dysfunction, Satellite Symposium of Diabetes and Atherosclerosis, Lyon, France

1997 Co-chairperson: Session on "Animal Models of Disease/Diabetes," American Heart Association, National Meeting, Orlando, Florida

1998 Co-chairperson: Session on "Diabetic Complications," American Diabetes Association, National Meeting, Chicago, Illinois

1999 Co-chairperson: Session on "Macrophage Activation and Scavenger Receptor Biology," Keystone conference, Inflammatory Paradigms and the Vasculature, Santa Fe, New Mexico

1999 Rapporteur, Session on "Vascular permeability in diabetes," Endothelial Cell Function in Diabetes Mellitus, The Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom

1999 Chairperson, Session on "Emerging Mechanisms of Diabetic Complications," American Diabetes Association, 59th Scientific Sessions, San Diego, California

1999 Co-chairperson and member of organizing committee, NIH/NIDCR-sponsored workshop on Diabetes and Oral Health, Washington, D.C.

Session chair, NIH/NIDCR-sponsored workshop on Diabetes and Oral Health, "Diabetes and Wound Healing," Washington, D.C.

2000 Co-Chairperson, Session on "Mechanisms and Diabetes and Atherosclerosis," American Heart Association, National Meeting, New Orleans, Louisiana

2001 Co-Organizer, Physicians & Surgeons Biomedical Sciences Symposium, "Angiogenesis," Arden House, Harriman, New York, July, 2001 &

Session chair: Tumor Biology, Key Roles for Angiogenesis and Lymphangiogenesis

2001 Session chair, 6th EASD/JDRF Oxford Workshop on the Molecular and Genetic Aspects of the Vascular Complications of Diabetes, session on Mechanisms of Vascular Disease, Keble College, Oxford, UK, August, 2001

2001 Co-Organizer, "The Diabetes Summit: A New Patient Treatment Regimen in Cardiovascular Disease", Anaheim, California, November, 2001

2002 Co-Chairperson, Annual Meeting of the American Heart Association, Session on Featured Research Session: Molecular Mechanisms in Atherosclerosis I; Subspecialty: Atherosclerosis/Hemostasis/Lipid Disorders, Chicago, Illinois, November, 2002.

2003 Discussion Leader, "How can we foster development of surrogate markers useful for clinical trials of potential new therapies?", Diabetes Mellitus Interagency Coordinating Committee, National Institutes of Health, Bethesda, Maryland

2003 Invited Participant, Working Group on the Cardiovascular Complications of Type 1 Diabetes, Sponsored by the Juvenile Diabetes Research Foundation International and the National Institutes of Health (NIDDK and NHLBI), Bethesda, Maryland

2003 Session chair, Adhesion Molecules and chemokines in atherogenesis, Workshop on Atherosclerosis: Molecular Basis of an Inflammatory Disease, Casteel Vaalsbroek, Vaals/Aachen, Germany

2003 Co-organizer, "Diabetic Complications: Progress through Animal Models," Sponsored by the National Institutes of Health (NIDDK, NHLBI, NINDS, NEI) & JDRFI, Bethesda, Maryland

2003 Session chair & discussion leader, "The Translation Pipeline: from the bench to the bedside,"

"Diabetic Complications: Progress through Animal Models," Sponsored by the National Institutes of Health (NIDDK, NHLBI, NINDS, NEI) & JDRFI, Bethesda, Maryland

2003 Co-Chairperson, Session on "Myocardial Ischemia-Associated Gene Expression," American Heart Association, National Meeting, Orlando, Florida

2004 Co-Chairperson, Session on "Inflammation & Tissue Injury," 12th International Congress of Immunology and 4th Annual Conference of FOCIS (Federation of Clinical Immunology Societies), Montreal, Canada

2004 Invited Participant, Diabetic Nephropathy Research Retreat, Sponsored by the National Institutes of Healthy and the American Society of Nephrology, Washington, D.C.

2005 Invited Participant, Meeting on the Special Statutory Funding Program for Type 1 Diabetes Research, Bethesda, Maryland

2005 Invited Participant, Meeting on Drug Screening for Hyperglycemic Cellular Injury, NIDDK/JDRF, Bethesda, Maryland

EDITORIAL SERVICE

1997 Associate (Guest) Editor, Journal of Gerontology

1998 Guest Editor, Investigative Ophthalmology and Visual Sciences

2003- Member, Editorial Board, Journal of Biological Chemistry

2004- Member, Editorial Board, Circulation

2004- Member, Editorial Board, Circulation Research

REVIEW COMMITTEES

1997 National Institutes of Health/National Institute of Dental Research, ad hoc reviewer, Special Emphasis Panel

1997 Wellcome Trust, London, England

1997 NIH/DRG: National Institues of Aging, ad hoc reviewer

1998 NIH/DRG: National Institues of Aging, ad hoc reviewer

1998 Special Review, University of Washington Diabetes Endocrinology Research Center (DERC) New Investigator Awards

1998 Reviewer, National Insitutes of Health, Request for Applications: "Pathogenesis and Therapy of Diabetic Complications"

1998 Endocrine Fellows Foundation, ad hoc reviewer

1999 Reviewer, Special Emphasis Panel, Program Project Grant, National Institute of Dental and Craniofacial Research

1999 Reviewer, Special Emphasis Panel, Program Project Grants, Mechanisms of Vascular Disease, National Heart, Lung and Blood Institute

1999 NIH/DRG: National Institutes of Aging, ad hoc reviewer

1999 Juvenile Diabetes Foundation International, ad hoc reviewer

1999 Reviewer, Special Emphasis Panel, National Institutes of Health, Request for Applications: "Pilot studies for new therapies for type 1 diabetes and its complications"

1999 Member, Vascular Biology I Study Section, American Heart Association

2000 Member, NIH/DRG:National Institutes of Aging, Biology of Aging- B

2000 National Institutes of Dental and Craniofacial Research, ad hoc reviewer

2000 Member, NIH Advisory Committee, Use of FY2001 Balanced Budget Act Funds for Type 1 Diabetes Research

2000-
2002 Member, Juvenile Diabetes Foundation International Medical Science Research Committe: Group III: Complications

2000 NIH/NIDDK/DRG: ad hoc reviewer

2001 Special Emphasis Panel (Chairperson), National Institute of Neurological Disorders and Stroke

2002 Ad hoc Member, Pathology A Study Section, Center for Scientific Review, National Institutes of Health

2002 Member, NIH/NIDDK Advisory Committee, Use of Special Congressional Funds for Type 1 Diabetes Research

2002 Reviewer, National Institutes of Aging, Site Visit and Review of Program Project Application

2002 Member, Ad hoc study section in response to a "Request for Applications," Bench to Bedside Therapy and Prevention of Diabetes and Its Complications, National Institutes of Health, NIDDK

2002-2003 Chair, Biology of Aging Study Section, NIA-B

2003 Chair, Special Emphasis Panel, National Institutes of Health

2004 Special Emphasis Panel, National Institute of Diabetes and Digestive and Kidney Diseases, RFA DK-03-019 "Bench to Bedside Research on type 1 diabetes and its complications"

2004 Special Review Committee, National Heart Lung & Blood Institute, Program Project Applicaton Review, Columbia, Maryland

2005 Special Emphasis Panel, Reviews of Cancer Centers of Excellence in Nanotechnology, National Cancer Institute, Washington, DC

2005 Ad hoc member, Vascular Cell and Molecular Biology study section, National Insitutes of Health, Bethesda, Maryland

BIBLIOGRAPHY

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2. Schmidt, A.M., Blum, R.H., Clayton, M., Speyer, J.L., Bottino, J., and Muggia, F.M. Phase II trial of cyclophosphamide and cis-platinum for non-small cell bronchogenic carcinoma. Am. J. Clin Oncol. 7:725-727, 1984.

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6. Schmidt, A.M., Yan, S.D., Brett, J., Mora, R., and Stern, D. Regulation of mononuclear phagocyte migration by cell surface binding proteins for advanced glycosylation endproducts. J. Clin. Invest. 92:2155-2168, 1993.

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10. Schmidt, A-M., Hasu, M., Popov, D., Zhang, J-H., Yan, S-D., Brett, J., Cao, R., Kuwabara, K., Costache, G., Simionescu, N., Simionescu, M., and Stern, D. The receptor for Advanced Glycation Endproducts (AGEs) has a central role in vessel wall interactions and gene activation in respons to AGEs in the intravascular space. PNAS(USA) 91:8807-8811, 1994.

11. Wautier, J-L., M-P. Wautier, A-M. Schmidt, G. M. Anderson, C. Zoukourian, L. Capron, O. Chappey, S-D. Yan, J. Brett, P-J. Guillausseau, and D. Stern. Advanced glycation endproducts (AGEs) on the surface of diabetic red cells bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. PNAS(USA) 91:7742-7746, 1994

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13. Kuwabara, K., D. Pinsky, A-M. Schmidt, C. Benedict, J. Brett, S. Ogawa, M. Broekman, A. Marcus, R. Sciacca, M. Michalak, F. Wang, Y-C. Pan, S. Grunfeld, S. Patton, T. Malinski, D. Stern, and J. Ryan. Calreticulin, an antithrombotic agent which binds vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine coronary arteries. J. Biol. Chem. 270:8179-8187, 1995.

14. Ritthaler, U., Y.Deng, Y. Zhang, J. Greten, M. Abel, J. Allenberg, G. Otto, H. Roth, A. Bierhaus, R. Ziegler, A-M. Schmidt, R. Waldherr, P. Wahl, D. Stern, and P. Nawroth. Expression of receptors for advanced glycation endproducts in peripheral occlusive vascular disease. Am. J. Pathol. 146: 688-694, 1995.

15. Schmidt, A-M., O. Hori, J. Chen, J.F. Li, J. Crandall, J. Zhang, R. Cao, S.D. Yan, J. Brett and D. Stern. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1): a potential mechanism for the accelerated vasculopathy of diabetes. J. Clin. Invest. 96:1395-1403, 1995.

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17. Abel, M., Ritthaler, U., Zhang, Y., Deng, Y., Schmidt, A.M., Greten, J., Sernau, T., Wahl, P., Andrassy,K., Ritz, E., Stern, DM., and P. Nawroth. Expression of receptors for advanced glycosylated end products in renal disease. Nephrology, Dialysis, Transplantation 10:1662-1667, 1995.

18. Wautier, J-L., C. Zoukourian, O. Chappey, M-P. Wautier, P-J. Guillausseau, R. Cao, O. Hori, D. Stern, and A.M. Schmidt. Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation endproducts blocks hyperpermeability. J. Clin. Invest. 97:238-243, 1996.

19. Schmidt, A.M., J. Crandall, R. Cao, O. Hori, and E. Lakatta. Elevated plasma levels of Vascular Cell Adhesion Molecule-1 (VCAM-1) in diabetic patients with microalbuminuria: a marker of vascular dysfunction and progressive vascular disease. Brit. J. Hematol.92:747 750, 1996.

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potential mechanism underlying accelerated periodontal disease associated with diabetes. J. Periodontal Res. 31:508-515, 1996.

21. Spanier, T., Oz, M., Levin, H., Weinberg, A., Moazami, N., Roberts, J.K., Mohr, J.P., Stern, D., Rose, E., and A.M. Schmidt. Activation of coagulation and fibrinolytic pathways in patients with Left Ventricular Assist Devices. J. Thoracic and Cardiovascular Surgery, 112:1090 1097, 1996.

22. Miyata, T., O. Hori, J.H. Zhang, S.D. Yan, L. Ferran, Y. Iida, and A.M. Schmidt. The Receptor for Advanced Glycation Endproducts (RAGE) mediates the interaction of AGE-b2 Microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway: implications for the pathogenesis of dialysis-related amyloidosis. J. Clin. Invest. 98:1088 1094, 1996.

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25. Zoukourian, C., Wautier, M., Chappey, O., Dosquet, C., Rohban, T., Schmidt, A.M., Stern, D., and Wautier, J.L. Endothelial cell dysfunction secondary to the adhesion of diabetic erythrocytes. International Andrology 15:195-200, 1996.

26. Yan, S-D., Zhu, H., Fu, J., Yan, S-F., Roher, A., Tourtellotte, W., Rajavashisth, T., Chen, X., Stern, D. and Schmidt, A-M. Amyloid-beta peptide-RAGE interaction elicits neuronal expression of M-CSF: a proinflammatory pathway in Alzheimer's disease. Proc. Natl. Acad. Sci. 94:5296-5301, 1997.

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31. Spanier, T., Oz, M., Minanov, O., Stern, D., Rose, E., and Schmidt, A.M. Active site- blocked Factor Ixa: a novel selective anticoagulant for use in cardiopulmonary bypass. Surgical Forum XLVIII:259-261, 1997.

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35. Hofmann, M.A., Kohl, B., Zumbach, M.S., Borcea, V., Bierhaus, A., Henkels, M., Amiral, J., Schmidt, A.M., Fiehn, W., Ziegler, R., Wahl, P., and Nawroth, P.P. Hyperhomocyst(e)inemia and endothelial dysfunction in IDDM. Diabetes Care 21:841-848, 1998.

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40. Spanier, T.B., Chen, J.M., Oz, M.C., Edwards, N.M., Kisiel,W., Stern, D.M., Rose, E.A., and Schmidt, A.M. Selective anticoagulation with active site-blocked Factor Ixa suggests separate roles for intrinsic and extrinsic coagulation pathways in cardiopulmonary bypass. J. Thoracic and Cardiovascular Surgery 116:860-869, 1998.

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II. Invited Articles/Chapters

1. Schmidt, A-M., Esposito, C., Brett, J., Ogawa, S., Clauss, M., Kirstein, M., Radoff, S., Vlassara, H., and Stern, D. Modulation of endothelial function and endothelial-monocyte interaction by advanced glycosylated end products of proteins. In Mononuclear Phagocytes, Ed. R. van Furth, Kluwer Academic Publishers (Dordrecht) pp. 202-207 1992.

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III. Abstracts

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165. Yan, S.D., Mei, L., Walker, D.G., Schmidt, A.M., Stern, D., and Lue, L. Amplification of the inflammatory response and increased amyloid deposition in double transgenic mice with targeted neuronal expression of mutant APP and microglial expression of RAGE. Abstract Book of the Annual Meeting of the Society for Neuroscience, Abstract # 23.10, 2004.

166. Rong, L.L., Adebayo, A., Lu, Y., Przedborski, S., Hays, A.P., Yan, S.F., and Schmidt, A.M. Microglial RAGE accelerates mortality and neuronal dysfunction in a murine model of familial amyotrophic lateral sclerosis. Abstract Book of the Annual Meeting of the Society for Neuroscience, Abstract # 706.2, 2004.

167. Wang, N., Wu, Z., Wang, C., Rong, L., Chen, X., Stern, D., Schmidt, A.M., and Yan, S.D. RAGE interaction with S100 activates phospho-p38, Akt and NF-kB in experimental autoimmune encephalitis (EAE) model. Abstract Book of the Annual Meeting of the Society for Neuroscience, Abstract # 936.11, 2004.

168. Guo, J.C., Qu, W., Ramasamy, R., Yan, S.F., D'Agati, V.D., and Schmidt, A.M. RAGE activates membrane-bound NADPH oxidase in podocytes via ERK1/2 MAP kinase. J. Am. Soc. Nephrol. 15:481a, 2004.

169. Zeng, S., Cataldegirmen, G., Feirt, N., Ippagunta, N., Dun, H., Qu, W., Lu, Y., Rong, L.L., Weinberg, A., Lefkowitch, J., Yan, S.F., Schmidt, A.M., and Emond, J.C. RAGE limits regeneration after massive liver injury by coordinated suppression of TNF-alpha and NF-kB Hepatology 40: #4 (Supplement): 284A, 2004.

170. Zeng, S., Ippagunta, N., Dun, H., Feirt, N., Qu, W., Yan, S.F., Schmidt, A.M., and Emond, J.C. Receptor for AGE (RAGE) dependent modulation of Egr-1 in total ischemia and reperfusion injury to the liver in a murine model. Hepatology 40: #4 (Supplement): 377A, 2004.

171. Hudson, B.I., Hofmann, M.A., Yang, Q., Harja, E., Kedia, P., Moser, B., Gregersen, P.K., Cupples, L.A., and Schmidt, A.M. The RAGE Gly82Ser polymorphism, atherosclerosis, thrombosis and the Framingham Offspring Study. FASEB J 19:387.9, 2005.

172. Hudson, B.I., Carter, A.M., Harja, E., Arriero, M., Yang, H., Moser, B., Grant, P.J., and Schmidt, A.M. A novel method for the detailed analysis of gene splice variants. FASEB J 19:523.7, 2005.

173. Harja, E., Hudson, B.I., Zou, Y.S., Lu, Y., Schmidt, A.M., and Fang, S.F. PKCbeta/egr- 1: a central axis in atherosclerosis. FASEB J 19:387.20, 2005.

174. Hudson, B.I., Hofmann, M.A., Yang, Q., Harja, E., Kedia, P., Moser, B., Gregersen, P.K., Cupples, L.A., and Schmidt, A.M. The RAGE Gly82Ser polymorphism and cardiovascular disease in the Framingham Offspring Study. Circulation 111:E225, 2005.

INVITED PRESENTATIONS

1. "Endothelial cell and mononuclear phagocyte receptors for advanced glycation endproducts," Gordon Research Conference, Vascular Biology, Colby Sawyer, New Hampshire, 1992.

2. "Cellular receptors for advanced glycation endproducts," American Heart Association Meeting, Mini-Symposium in Thrombosis and Hemostasis, New Orleans, Louisiana, 1992.

3. "Cellular receptors for advanced glycation endproducts: implication for endothelial and monocyte dysfunction in the pathogenesis of vascular lesions," Atherosclerosis Symposium, University of Regensburg, Germany, 1993.

4. "Cellular receptors for glycated proteins: implications for vascular dysfunction in atherosclerosis and diabetes," FASEB meeting, New Orleans, Louisiana, 1993.

5. "Cellular receptors for advanced glycosylation endproducts: implications for vascular disease in diabetes," Scientific Conference on the Molecular Biology of the Vascular Wall, American Heart Association, Boston, Massachusetts, 1993.

6. "Cellular receptors for advanced glycation endproducts: implications for vascular disease in atherosclerosis and diabetes," Research Seminar, National Institutes of Aging, National Institutes of Health, Baltimore, Maryland, March, 1994.

7. "Cellular receptors for advanced glycation endproducts: implications for vascular dysfunction in atherosclerosis and diabetes," Grand Rounds, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York, March, 1994.

8. “Atherosclerosis, aging and diabetes: common mechanisms, “Minisymposium on Vascular Permeability, FASEB, Anaheim, California, April 1994.

9. “Glycated proteins and their receptors in vascular disease,” Grand Rounds, Department of Cardiology, UCLA School of Medicine, Los Angeles, California, April 1994.

10. “Advanced Glycation Endproducts and their cellular receptor: implications for diabetic vascular disease, Endocrinology Grand Rounds, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York, January, 1996.

11. “AGE-receptor interaction: implications for accelerated atherosclerosis observed in diabetes, Cardiology Grand Rounds, Department of Medicine, New York University School of Medicine, New York, New York, February, 1996.

12. “AGE-RAGE cellular interaction: implications for the development of diabetic complications,” Nephrology Grand Rounds, Department of Medicine, Downstate Medical Center, Brooklyn, New York, May, 1996.

13. “RAGE in atherosclerosis and Alzheimer’s disease,” Clinical Research Seminars, Rockefeller University, New York, New York, June, 1996.

14. “RAGE: implications for complications of diabetes,” Grand Rounds, Department of Medicine, Division of Nephrology, North Shore University Hospital, Manhasset, New York, September, 1996.

15. “AGE-RAGE interaction: implications for the development of diabetic complications,” Grand Rounds, Department of Pediatrics, Columbia University College of Physicians and Surgeons, New York, New York, October, 1996.

16. “The receptor for advanced glycation endproducts: implications for the pathogenesis of diabetic complications,” Scientific congress on the vascular endothelium: basic and clinical aspects, Pisa, Italy, November, 1996.

17. “Receptor for AGE, RAGE: implications for the biology of aging,” National Institutes of Aging and the Glenn Foundation workshop on “Molecular aspects of age-related cardiovascular decline,” Montecito, California, January, 1997.

18. “Interaction of Advanced Glycation Endproducts (AGEs) with their cellular receptor RAGE: implications for vascular and inflammatory cell dysfunction in diabetes,” Symposium of the Baker Medical Research Institute on “Atherosclerosis and the Vessel Wall,” Melbourne, Austrailia, February, 1997.

19. “Prevention of diabetic complications,” 10th annual congress, Mexican Diabetes Federation, Aguascalientes, Mexico, March, 1997.

20. “Advanced Glycation Endproducts (AGEs) in diabetic periodontal disease,” Sunstar Chapel Hill Symposium, Periodontal diseases and human health, Chapel Hill, North Carolina, March, 1997.

21. “RAGE and diabetic atherosclerosis,” Annual Scholar’s Day Program, Council for Tobacco Research, New York, New York, April, 1997.

22. “RAGE and the pathogenesis of diabetic complications,” Seminar, Center for Transgene Technology and Gene Therapy, Leuven, Belgium, May, 1997.

23. “Interaction of glycated proteins with the vessel wall: implications for the pathogenesis of accelerated atherosclerosis in diabetes,” 29th annual Hugh Lofland Conference on atherogenesis and the vessel wall, Honolulu, Hawaii, June, 1997.

24. “AGEs and RAGE: implications for the pathogenesis of diabetic complications,” Invited speaker, Symposium on Endothelial Dysfunction in Diabetes, annual meeting, American Diabetes Association, Boston, Massachusetts, June, 1997.

25. “Interaction of Advanced Glycation Endproducts (AGEs) with their receptor RAGE: implications for the biology of aging,” 1997 World Congress of Gerontology, 16th Congress of the International Association of Gerontology, Adelaide, Australia, August, 1997.

26. “RAGE and vascular cell dysfunction,” Juvenile Diabetes Foundation and European Association for the Study of Diabetes: Workshop on Diabetic Retinopathy, Oxford, England, September, 1997.

27. “Advanced Glycation Endproducts and RAGE: Implications for enhanced oxidant stress in the pathogenesis of complications in diabetes and beyond,” 4th Kobe Study Group of Vascular Medicine: Cross Talk between NO and Oxygen Radicals, Kobe, Japan, September, 1997.

28. “Interaction of Advanced Glycation Endproducts with their cellular receptor RAGE: implications for the pathogenesis of complications in diabetes and beyond,” Center for Blood Research, Harvard University, Boston, Massachusetts, September, 1997.

29. “Interaction of advanced glycation endproducts with their cellular receptors,” Symposium, Diabetetes and Endothelial Dysfunction, Lyon, France, October, 1997.

30. “AGEs and RAGE: Implications for the pathogenesis of diabetic complications,” Grand Rounds, Department of Medicine, New York University School of Medicine, New York, New York, October, 1997.

31. “Selective Anti-thrombotic therapy without interfering with protective hemostasis: role of Factor IX/IXa,” Frontiers in Translational and Clinical Research: Anti-Coagulation: Present and Future, Columbia University College of Physicians and Surgeons, New York, New York, November, 1997.

32. “AGEs and RAGE: Implications for the pathogenesis of complications in diabetes and beyond,” Seminar, Department of Physiology and Cellular Biophysics, Columbia University College of Physicans and Surgeons, New York, New York, November, 1997

33. “AGEs and RAGE: Implications for the pathogenesis of complications in diabetes, atherosclerosis and beyond,” Seminar, Novartis, Summit, New Jersey, December, 1997

34. “RAGE: A novel target for the therapy of complications in diabetes and beyond,” Invited Scholar lecture, Department of Dermatology, Columbia University College of Physicians and Surgeons, New York, New York, Janaury, 1998.

35. “AGEs and RAGE: Implications for vascular complications in diabetes,” Keystone symposium on the Endothelium, Lake Tahoe, Nevada, March, 1998.

36. “Receptor for AGE: Implications for the pathogenesis of complications in diabetes,” Diabetes Research Seminar, Case Western University School of Medicine, Cleveland, Ohio, May, 1998.

37. “Receptor for Advanced Glycation Endproducts (AGE) and implications for the pathogenesis of diabetic complications ”, New York/New Jersey Molecular Biology Club, New Jersey Medical School, Newark, New Jersey, May, 1998.

38. “Active site-blocked Factor IXa in Cardiac Surgery,” Cambridge Healthech Institute symposium on novel anticoagulants, San Diego, California, May, 1998.

39. “Receptor for AGE (RAGE): Novel insights into Diabetes and Inflammation,” Department of Pediatrics Grand Rounds, Columbia University College of Physicians and Surgeons, August, 1998.

40. “RAGE and the pathogenesis of vascular complications in diabetes,” Xth International Vascular Biology meeting, Cairns, Australia, August, 1998.

41. “Heparin and its alternatives,” Annual meeting, Extracorporeal Life Support Organziation, San Antonio, Texas, September, 1998.

42. “Suppression of accelerated diabetic atherosclerosis by soluble RAGE (sRAGE),” The Vascular Endothelium: Basic and Clinical Aspects, Second International Congress, Pisa, Italy, October, 1998.

43. “AGE receptors and oxidative stress,” Diabetic Complications Conference, Joint Symposium in celebration of the Joslin Diabetes Center’s 100th anniversary, Boston, Massachusetts, October, 1998.

44. “Receptor for AGE, RAGE: Implications for chronic complications in diabetes and inflammation,” Whitaker Cardiovascular Institute Seminar, Boston University School of Medicine, Boston, Massachusetts, January, 1999.

45. “Receptor for AGE (RAGE): “Novel Proinflammatory Ligands and Insights into Inflammation,” Keystone Conference, Inflammatory Paradigms and the Vasculature, Santa Fe, New Mexico, February, 1999

46. "RAGE and implications for chronic complications in diabetes and inflammation,” Bergen Community Regional Blood Center, Paramus, N.J., March, 1999.

47. "Receptor for AGE: implications for the pathogenesis of complications in diabetes and inflammation," New York Metro Pediatric Endocrine Society, N.Y., N.Y., April, 1999.

48. "Advanced Glycation Endproducts and atherosclerosis," FASEB summer conference on Thrombin and Vascular Medicine, Saxton River, Vermont, June, 1999.

49. "Receptor for AGE (RAGE): Implications for Vascular and Inflammatory Dysfunction in Diabetes and other Disorders," Gordon Research Conference on "Angiogenesis and Microcirculation," Salve Regina University, Newport, Rhode Island, August, 1999.

50. "Vascular and endothelial dysfunction in diabetes," Plenary session, The Fourth International Diabetes Federation, Western Pacific Region Congress, Sydney, Australia, August, 1999.

51. "Markers of vascular and endothelial dysfunction in diabetes, " "Meet the Professor session," The Fourth International Diabetes Federation, Western Pacific Region Congress, Sydney, Australia, August, 1999.

52. "Present status of the AGE receptors: RAGE and future developments," ENGAGE meeting," European Association for the Study of Diabetes, Brussels, Belgium, September, 1999.

53. "Receptor for AGE (RAGE): Implications for chronic cellular dysfunction in diabetes, inflammation and tumor biology," Grand Rounds, Division of Rheumatology, Department of Medicine, New York University School of Medicine, October, 1999.

54. "The Molecular Pathogenesis of Diabetic Complications," Frontiers in Diabetes Research, The Naomi Berrie Diabetes Center, Columbia University, New York, New York, November, 1999.

55. "Role of Advanced Glycation End-products in the clinical complications of diabetes," Jubilee symposium in honour of Professor Bernard Jacotot, The French Atherosclerosis Society, Paris, France, November, 1999.

56. "Advanced Glycation Endproducts and their receptors," NIH/NIDCR-sponsored workshop on Diabetes and Oral Health, Washington, D.C., December, 1999.

57. "Advanced Glycation Endproducts and their Receptor RAGE: Implications for the pathogenesis of complications in diabetes, inflammation, Alzheimer's disease and cancer," Institute for Biochemistry, Justus-Liebig-University, Gießen, Germany, December, 1999.

58. "AGE-RAGE interaction: implications for the development of diabetic vasculopathy," Renal Grand Rounds, The New York Hospital Medical Center of Queens, " Queens, New York, March, 2000.

59. "Receptor for Advanced Glycation Endproducts (RAGE) and implications for diabetic complications, inflammation and tumor biology," Lung Biology Conference, Division of Pulmonary Medicine, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, March, 2000.

60. "Receptor for AGE (RAGE) is a gene within the major histocompatibility class III region: implications for host response mechanisms in homeostasis and chronic diseases," Immunology Seminar Program, College of Biological Sciences, Ohio State University School of Medicine, April, 2000.

61. "Receptor for AGE (RAGE) and implications for the pathogenesis of diabetic complications, inflammation and cancer," Distinguished Lecture, Department of Oral Biology, State University of New York at Buffalo School of Dentistry, Buffalo, New York, May, 2000.

62. "Receptor for AGE (RAGE) and implications for the pathogenesis of diabetic complications and inflammation," German Diabetes Association, Munich, Germany, May, 2000.

63. "Receptor for AGE: a multiligand receptor of the immunoglobulin superfamily with implications for the pathogenesis of diabetic complications and other disorders," Current Topics in Glycobiology, Helsinki, Finland, June, 2000.

64. "Blockade of RAGE, a New Approach to the Treatment of the Complications of Diabetes," Juvenile Diabetes Research Foundation, New York, New York, October, 2000.

65. "RAGE: updates on tumor biology and inflammation paradigms," Department of Medicine, Faculty Research Seminar, Columbia University, New York, New York, December, 2000.

66. "RAGE - a multiligand tale," Seminar, Naomi Berrie Diabetes Center, Columbia University, New York, New York, December, 2000.

67. "RAGE and peripheral nerve repair," Keystone Symposium on Neuronal and Vascular Stress: a New Window on Alzheimer's Disease, Durango, Colorado, January, 2001.

68. "RAGing against the complications of diabetes," Juvenile Diabetes Research Foundation International, Meeting of the Board of Directors, Tampa, Florida, February, 2001.

69. "RAGE and the complications of diabetes and inflammation," Seminar, Boston University Goldman School of Dental Medicine, Boston, Massachusetts, April, 2001.

70. "The Role of Advanced Glycation Endproducts (AGE) and their receptor RAGE in Diabetes, The Periodontal-Systemic Connection: A State of the Art Symposium, Sponsored by the NIDCR and the AAP, Bethesda, Maryland, April, 2001.

71. "RAGE: Updates on the Amyloidoses and Inflammation," Seminar, Department of Molecular Medicine, Weill-Cornell University Medical College, New York, New York, April, 2001.

72. "RAGE and the complications of diabetes: inflammatory overtones," 6th EASD/JDRF Oxford Workshop on the Molecular and Genetic Aspects of the Vascular Complications of Diabetes, Keble College, Oxford, UK, August, 2001.

73. "The Current RAGE of Diabetes," The Diabetes Summit: A New Patient Treatment Regimen in Cardiovascular Disease, Anaheim, California, November, 2001.

74. "RAGE and the Complications of Diabetes - Insights into Proinflammatory Mechanisms," Invited Speaker, Meeting of the Oral Biology, Immunology and Microbiology Research Group, Longboat Key, Florida, January, 2002.

75. "RAGE: Implications for Diabetic Complications and Beyond," Biochemical Pharmacology Discussion Group, New York Academy of Sciences, New York, New York, January, 2002.

76. "RAGE and the complications of diabetes and inflammation," Seminar, Department of Clinical Pharmacology, Department of Medicine, New York University School of Medicine, March, 2002.

77. "RAGE: insights into proinflammatory mechanisms in diabetes and immune/inflammatory disorders," Keystone Symposium, "Inflammatory Paradigms and the Vasculature II," Steamboat Springs, Colorado, April, 2002.

78. "RAGE; insights into the pathogenesis of diabetic complications and beyond," Grand Rounds, Department of Medicine, College of Physicians & Surgeons, Columbia University, New York, New York, April, 2002.

79. "RAGE and the complications of diabetes," Keynote Lecture, Banting and Best Diabetes Centre Annual Scientific Day, University of Toronto, Toronto, Canada, May, 2002.

80. "RAGE blockade and implications for the treatment of diabetic complications, inflammation, neurodegenerative disorders and cancer: a quest for clinical translation," Grand Rounds, Department of Surgery, College of Physicians & Surgeons, Columbia University, New York, New York, June, 2002.

81. "AGE, RAGE and Animal Models of Diabetic Complications," Invited Speaker, Animal Models of Diabetic Complications, National Institutes of Diabetes, Digestive and Kidney Disease, Arlington, Virginia, August, 2002.

82. "Receptor for AGE (RAGE) and Implications for Diabetic Complications, Tumors and Beyond," Department of Medicine, Grand Rounds, University of Vermont, October, 2002.

83. "Receptor for AGE (RAGE): a quest for clinical translation," Seminars in Investigative Medicine, University of Vermont, October, 2002.

84. "Receptor for AGE (RAGE): Implications for Diabetic Complications, Tumors and Beyond, Seminar, Department of Biochemistry, University of Helsinki, Helsinki, Finland, October, 2002.

85. "Diabetic Vascular Oxidant Stress," Invited Presentation, Session on Molecular Mechanisms of Atherosclerotic Vascular Disease in type 2 Diabetes," Annual meeting of the American Heart Association, Chicago, Illinois, November, 2002.

86. "RAGE and the Vascular Complications of Diabetes," Invited Speaker, Alfediam (Association de Langue Francaise Pour L'Etude Du Diabete Et Des Maladies Metaboliques): Meeting on "Atherosclere et Diabete: Acquis et Defis", Pasteur Institute, Paris, France, December, 2002.

87. "RAGE, diabetes and the inflammatory response," Seminar, Division of Rheumatology, Department of Medicine, College of Physicians & Surgeons, Columbia University, New York, New York, December, 2002.

88. "RAGE and the complications of diabetes and beyond," Seminar, Department of Microbiology and Immunology, University of Western Ontario, Ontario, Canada, January, 2003.

89. "RAGE and the complications of diabetes," Seminar, Naomi Berrie Diabetes Center, College of Physicians & Surgeons, Columbia University, New York, New York, February, 2003.

90. "Understanding Diabetes- It's All in the RAGE," Myocardial Reperfusion XVI: Concepts and Controversies," American College of Cardiology, Chicago, Illinos, March, 2003.

91. "RAGE-dependent mechanisms and metabolic imprinting in the pathogenesis of diabetic complications," 20th Anniversary Symposium, Metabolic Imprinting and the Long-Term Complications of Diabetes Mellitus: Bench to Bedside and Back, National Institutes of Health, Bethesda, Maryland, April, 2003.

92. "RAGE and the Complications of Diabetes," Seminar, Diabetes Research Center, Albert Einstein College of Einstein, Bronx, New York, April, 2003.

93. "RAGE and the complications of diabetes and inflammation," Invited Speaker, Symposium on "Evolving Epidemic of Diabetes and Vascular Disease," University of Virginia, Charlottesville, Virginia, May, 2003.

94. "RAGEing against the complications of diabetes," Invited speaker, Annual meeting of the Northern New Jersey/Rockland County Chapter of the Juvenile Diabetes Foundation International," Tenafly, New Jersey, June, 2003.

95. "RAGE and amplification of proinflammatory pathways in the immune response," Invited Speaker, Arthritis Research Conference, Arthritis Foundation, Keystone, Colorado, June, 2003.

96. "Insights into Pathogenic Mechanisms in Diabetic Atherosclerosis and Cardiac Dysfunction," 8th European Association for the Study of Diabetes/Juvenile Diabetes Research Foundation Oxford Workshop, Keble College, Oxford, United Kingdom, August, 2003.

97. "RAGE and vascular inflammation: insights into the vascular complications of diabetes," Workshop on Atherosclerosis- Molecular Basis of an Inflammatory Disease, Casteel Vaalsbroek, Vaals/Aachen, Germany, September, 2003.

98. "RAGE: Moving to the Clinic for the Cardiovascular Complications of Diabetes," Workshop entitled: "Diabetic Complications: Progress through Animal Models," Sponsored by the National Institutes of Health (NIDDK, NHLBI, NINDS, NEI) & JDRFI, Bethesda, Maryland, October, 2003.

99. "Systemic Markers of Inflammation," Invited Speaker, Type 2 Diabetes, the Metabolic Syndrome and Obesity: Evolving the Paradigms, Mc Lean, Virginia, January, 2004.

100. "Interaction between aldose reductase and RAGE-AGE pathways in diabetic myocardium," Invited Speaker, International Polyol Pathway Conference, Kona, Hawaii, March, 2004.

101. RAGing against the complications of diabetes: new directions and future therapies," Invited Speaker, International Polyol Pathway Conference, Kona, Hawaii, March, 2004.

102. "RAGE and the Complications of Diabetes and Beyond: Inflammation, Tumors and Innate Functions," Department of Biology Seminar, New York University, New York, New York, March, 2004.

103. "AGEs and RAGE as Therapeutic Targets in Diabetes," Invited Speaker, American Society of Hypertension, New York, New York, May, 2004.

104. "RAGE and the cardiovascular complications of diabetes," Grand Rounds, Division of Cardiology, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, May, 2004.

105. "All the RAGE," Invited Speaker, Session on Mechanisms of Vascular Wall Damage, 64th annual sessions of the American Diabetes Association, Orlando, Florida, June, 2004.

106. "RAGE: The Complications of Diabetes and Neurodegenerative Disorders: Mechanisms & Therapeutic Strategies," Grand Rounds, Invited Speaker, Department of Neurology, Columbia University Medical Center, New York, New York, June, 2004.

107. "Receptor for AGE (RAGE) is a multiligand receptor of the immunoglobulin superfamily: implications for modulation of the inflammatory response," Session on "Inflammation & Tissue Injury," 12th International Congress of Immunology and 4th Annual Conference of FOCIS (Federation of Clinical Immunology Societies), Montreal, Canada, July, 2004.

108. "Receptor for AGE (RAGE): a multiligand receptor of the immunoglobulin superfamily- implications for the pathogenesis of diabetic complications," Invited Speaker, Plenary Session, 8th International Symposium on the Maillard Reaction," Charleston, South Carolina, August, 2004.

109. "Receptor for Advanced Glycation Endproducts: Insights into the pathogenesis of diabetic complications," 5th Annual Rachmiel Levine Symposium: Advances in Diabetes Research From Cell Biology to Cell Therapy, Los Angeles, California, October, 2004.

110. "RAGE Blockade: From Mice to Man- moving to the clinic," Advances in Translational Research, Columbia University Medical Center New York Presbyterian Hospital and the Science Office of the Embassy of Italy, New York, New York, October, 2004.

111. "RAGE: Diabetic Complications and the Inflammatory Response," Society for Biomaterials: "Biomaterials in Regenerative Medicine: The Advent of Combination Products," Philadelphia, Pennsylvania, October, 2004.

112. "AGE, RAGE & Diabetic Complications," The Pfizer Carousel of Hope Diabetes Symposium on "Inflammation: Cause And Consequence of Diabetes and Vascular Complications," Beverly Hills, California, October, 2004.

113. "RAGE: Implications for Diabetic Complications, Inflammation, Neurodegeneration and Tumors," Biogen, Inc., Boston, Massachusetts, November, 2004.

114. "RAGE & the Cardiovascular Complications of Diabetes," Invited Speaker, Session on Diabetes and Cardiovascular Disease, Annual Meeting of the American Heart Association, November, 2004.

115. "Glycation, Inflammation and the Complications of Diabetes: The RAGE Connection," Endocrinology Canada International Symposium, The Science of Diabetes Complications, Implications for Novel Therapy, Toronto, Canada, November, 2004.

116. "RAGE & the Complications of Diabetes, Inflammation and Cancer," Department of Anesthesia Case Conference and Guest Lecture Series, Columbia University, New York, New York, January, 2005.

117. "RAGE & the Complications of Diabetes and Inflammation," Seminar, Ewha University, Seoul, Korea, February, 2005.

118. "RAGE, Diabetes and Inflammation: Round & Round We Go," Invited speaker, Session on Pathophysiology of the Metabolic Syndrome, Annual Meeting of the American College of Cardiology, Orlando, Florida, March, 2005.

119. "RAGE, Inflammation and Diabetes: Insights into Complications," Seminar Speaker, Biomedical Seminar Series, Penn State University, College of Medicine, Hershey, Pennsylvania, April, 2005.

120. "RAGE and the Complications of Diabetes and Inflammation," Invited Speaker, Type 1 Diabetes, Naomi Berrie Diabetes Center, Columbia University, New York, New York, May, 2005.

121. "RAGE and the complications of diabetes- the role of inflammation." Invited Speaker, Symposium on "Genetics and Inflammatory Mechanisms in Cardiovascular Disease," Downstate Medical Center, Brooklyn, New York, June, 2005.

122. "RAGE and diabetes – to the heart of the matter." Invited Speaker, American Heart Association Basic Cardiovascular Science Symposium on Targeting Heart Failure: New Science, New Tools, New Strategies, Keystone, Colorado, July, 2005.

123. Discussion Leader, Gordon Research Conference on Assisted Circulation, Managing Adverse Events, Big Sky, Montana, August, 2005.

124. "The Receptor for Advanced Glycation Endproducts and the Vascular Complications of Diabetes," European Society for Pediatric Endocrinology, ESPE/LWPES 7th Joint meeting, Lyon, France, September, 2005.

125. "RAGE and the pathogenesis of complications in types 1 and 2 diabetes," Workshop sponsored by the American Diabetes Association and the Juvenile Diabetes Research Foundation International, Boston, Massachusetts, September, 2005.

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gap-like genes is therefore consistent with the proposed origin of the gene from the HOX-cluster[3]. By adopting BTD as a partner, EMS could escape phenotypic suppression by gnatho-cephalic HOX gene activities and specify the intercalary head segment identity. □

Methods

Drosophila strains

We used Oregon R, btd^{XG}, svb^{YP17b} btd^{XG} (refs 7,19), homozygous lines of the transgenes described below and hsp70-BTD/hsp70-BTD; ems[1]/+ for heat-shock experiments in an *ems* mutant background. *svb btd* double mutant was used to identify *btd* mutant cuticles[19].

Generation and analysis of transgenic animals

$VP16^{BTDzf}$, N-BTD, C-BTD, N-BTDΔU, N-BTDΔS/T and N-BTDΔQ were constructed by polymerase chain reaction (PCR) and standard cloning procedures. N-BTD lacks amino acids 448–644 of the *btd* sequence[7], C-BTD lacks 1–311, N-BTDΔU lacks 240–326 and 448–644, N-BTDΔS/T lacks 116–240 and 448–644, and N-BTDΔQ lacks 6–116 and 448–644. After sequencing, constructs were cloned into a P-element vector providing the 5.2 kilobases (kb) *btd cis*-acting element[13]. btd–EMS contains the 2.2 kb *XbaI–EcoRV* fragment of *ems* cDNA[8,9], UAS–EMS contains a 2.2 kb *EcoRI* fragment of *ems* cDNA in pUAST (ref. 15), and hsp70–BTD contains a 3.1 kb genomic *btd BamHI* fragment in the *BglII* site of pCaSpeR-hs (ref. 20).

To generate transgenic flies, constructs were injected in *white* mutant embryos[21]. Except for N-BTDΔQ, at least two independent transgenic lines (balanced over CyO or TM3) were examined. Immunological stainings of embryos[22] were performed with anti-β-galactosidase (Cappel), FP3.38 anti-UBX (ref. 23), 4D9 anti-EN (Developmental Studies Hybridoma Bank; University of Iowa)[22] and 22C10 (ref. 24) primary antibodies using the Vectastain ABC Elite Kit (Vector). Homozygous mutant embryos were identified through blue balancers. Stained embryos were embedded (Canada Balsam, Sigma) or drawn into capillaries. Embryos (30-min collections) were heat-shocked (1 h; 37 °C) after 2 h of development (25 °C). Cuticle preparations[19] and embryos were photographed with a Zeiss Axiophot.

Protein binding assays

Full-length *ems* cDNA[8,9] was cloned into a baculovirus transfer vector[25] to generate a flag-tag fusion construct for overproduction of EMS; the BTD and Sp1 constructs are described[16]. Recombinant baculovirus (Baculogold viral DNA, Pharmingen), expression and purification of Flag-epitope-tagged proteins from Sf9 cells were described[25]. C-BTDΔzf refers to the 880-bp carboxy-terminal *BglI SspI btd* fragment cloned into *PvuII*-digested pRSETB (Invitrogen). For protein interaction studies, about 50 ng of Flag-epitope-tagged proteins (immobilized on Flag-M2 antibody resin; Eastman Kodak) were incubated (3 h, 4 °C) with [^{35}S]methionine-labelled proteins generated by the TNT-coupled *in vitro* transcription/translation system (Promega), washed extensively with 50 mM HEPES, pH 7.9, 25 mM $MgCl_2$, 40% glycerol, 0.8 M KCl, 1% Triton X-100, separated by SDS–PAGE and visualized by autoradiography.

Yeast two-hybrid assays were performed as described (Clontech manuals: Yeast Protocols Handbook; MATCHMAKER Two-Hybrid System 3). EMSΔHD–BD (residues 1–383) was generated by inserting an *EcoRI/BamHI* fragment taken from EMSΔHD–AD into pGBKT7. EMSΔHD–AD was PCR-amplified from *ems* cDNA[8,9] (primers: EMS1F: 5′-CCCGAATTCATGACTAAGACGATTCCG-3′; EMS1R: 5′-CCGCCCGGGCTAGGGCACCAGGAAACTTCC-3′), BTD1–AD (resides 1–217) and BTD2–AD (residues 105–424) were PCR-amplified from *btd* cDNA[7] (primers: BTD1F: 5′-CGCGAATTCATGATCGATGCGGCCTGC-3′; BTD1R: 5′-GCCGGGCCCTACGCCGCAGCTGCTGCTGCC-3′ and BTD2F: 5′-GCCGAATTCCTATCCGGCTCGAGTTCC-3′; BTD2R: 5′-CGCGGGCCCTAGGCGGCCAGTACCTTCTTGC-3′, respectively). PCR fragments were cloned into *EcoRI/SmaI*-digested pGADT7. N-BTD–AD (residues 1–424) was created by opening BTD1–AD with *StuI/BamHI* and inserting an *StuI/BamHI* fragment from BTD2–AD. C-BTD–AD (residues 405–645) was PCR-amplified (primers were BTD3F: 5′-GGCGGCATATGAGCGATCACCTCAGC-3′; BTD3R: 5′-CCCGGGCCCATCCTAGGCGGTGGC-3′) and cloned into *NdeI/SmaI*-digested pGADT7.

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Blockade of RAGE–amphoterin signalling suppresses tumour growth and metastases

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The receptor for advanced glycation end products (RAGE), a multi-ligand member of the immunoglobulin superfamily of cell surface molecules[1,2], interacts with distinct molecules implicated in homeostasis, development and inflammation, and certain diseases such as diabetes and Alzheimer's disease [3–8]. Engagement of RAGE by a ligand triggers activation of key cell signalling

pathways, such as $p21^{ras}$, MAP kinases, NF-κB and cdc42/rac, thereby reprogramming cellular properties[9–11]. RAGE is a central cell surface receptor for amphoterin, a polypeptide linked to outgrowth of cultured cortical neurons derived from developing brain[5,12–15]. Indeed, the co-localization of RAGE and amphoterin at the leading edge of advancing neurites indicated their potential contribution to cellular migration, and in pathologies such as tumour invasion. Here we demonstrate that blockade of RAGE–amphoterin decreased growth and metastases of both implanted tumours and tumours developing spontaneously in susceptible mice. Inhibition of the RAGE–amphoterin interaction suppressed activation of p44/p42, p38 and SAP/JNK MAP kinases; molecular effector mechanisms importantly linked to tumour proliferation, invasion and expression of matrix metalloproteinases[16–23].

Amphoterin and RAGE are expressed in a range of cell types[15,24], and RAGE–amphoterin modulates cellular invasive properties in developing neurons. Thus, it was logical to examine the potential role of these molecules in tumour biology. Rat C6 glioma cells[15] provided an ideal starting point for these studies, as immunoblotting of C6 glioma cell lysates demonstrated expression of both RAGE (Fig. 1a, lane 3) and amphoterin (Fig. 1b, lane 3). Confocal microscopy confirmed the co-localization of RAGE and amphoterin in these cells (see Fig. 1 in Supplementary Information). The absence of another RAGE ligand, EN-RAGE[8], in C6 glioma (Fig. 1c, lanes 3 and 5) led us to pursue potential roles for amphoterin and RAGE in tumour behaviour.

We focused on strategies to inhibit the function of RAGE and amphoterin *in vivo*. The extracellular region of RAGE is composed of one 'V'-type followed by two 'C'-type immunoglobulin-like domains and comprises the soluble, ligand-binding domain[2]. Following the extracellular and transmembrane spanning domains is a short, highly charged cytosolic tail, which is essential for RAGE-dependent intracellular signalling and subsequent cellular activation[8]. Therefore, four strategies for blocking RAGE–amphoterin-mediated cellular stimulation *in vivo* are: (1) administration of soluble, extracellular ligand-binding domain of RAGE, sRAGE; (2) administration of blocking $F(ab')_2$ fragments derived from anti-RAGE and/or anti-amphoterin IgG; (3) generation of stably transfected C6 glioma expressing a RAGE mutant devoid of the cytosolic tail, so that the truncated form of the receptor is present on the cell surface and competent for ligand binding, but exerts a dominant-negative effect on RAGE signalling; and (4) generation of stably transfected C6 glioma expressing sRAGE.

Administration of sRAGE once daily[4,5] to immunocompromised (athymic nude) mice upon injection of rat C6 glioma cells caused dose-dependent decreases in tumour volume (Fig. 2a). As amphoterin and RAGE were both present in the tumour bed, we assessed their effects by using monospecific polyclonal rabbit $F(ab')_2$ fragments prepared from antibodies to RAGE and/or amphoterin. These antibody fragments were administered to immunocompromised (severe combined immunodeficiency; SCID) mice from the time of inoculation with C6 glioma cells. Compared with SCID mice receiving nonimmune $F(ab')_2$ or anti-EN-RAGE $F(ab')_2$, animals treated with anti-amphoterin or anti-RAGE $F(ab')_2$ had a significant reduction in tumour volume after 21 days (Fig. 2b). Simultaneous treatment with both anti-RAGE and anti-amphoterin $F(ab')_2$ resulted in greater reduction in tumour volume compared with treatment with the control $F(ab')_2$ or either antibody alone (Fig. 2b).

A limitation of these modalities was the possibility that such proteins administered intraperitoneally would not reach sufficiently high levels in the tumour bed to inhibit the receptor. Therefore, we stably transfected rat C6 glioma cells with tail-deletion RAGE (T), soluble RAGE (S) and full-length RAGE (F), or mock-transfected them (M). Three independent clones from each transfection were implanted into immunocompromised (athymic nude) mice to evaluate the role of RAGE in tumour growth. Analysis of the resulting tumours by immunoblotting revealed the following increase in RAGE expression compared with mock-transfected clones: clones F1–3: 11.7-, 9.0- and 22.6-fold; clones T1–3: 20.2-, 7.7- and 9.4-fold; and clones S1–3: 3.4-, 7.0- and 8.4-fold. Compared with mock-transfectants ($n = 17$) (Fig. 2c, d), about a fivefold increase in tumour volume was observed in cells overexpressing full-length RAGE on day 21 ($n = 18$) (Fig. 2c, e). Tumour volume was decreased about threefold in tumours derived from C6 glioma clones transfected with the tail-deletion mutant ($n = 21$) (Fig. 2c, f). Tumour volume also decreased (about 6.5-fold) in neoplasms derived from sRAGE-transfected C6 glioma ($n = 18$) (Fig. 2c, g). These results indicate the involvement of RAGE-mediated cellular activation in tumour growth and phenotype.

To delineate the mechanisms by which blockade of RAGE suppressed tumour growth, we analysed implanted tumours over time. On days 1, 3 and 7, mock- and full-length RAGE-transfected C6 glioma had begun to grow and invade surrounding muscle and connective tissue. In contrast, transfectants expressing tail-deletion RAGE or sRAGE on days 1, 3 and 7 were limited to the immediate area where they had been injected. Only at day 14 did tail-deletion RAGE transfectants and sRAGE-transfectants begin to invade the surrounding tissues (see Fig. 2 in Supplementary Information). Consistent with these observations, cells expressing glial fibrillary acidic protein (GFAP) at the centre of the tumour increased in area in full-length RAGE-transfected clones on days 3, 7, and 14 as compared with mock-transfected tumours (Fig. 3a). In contrast, tumour cells expressing tail-deletion RAGE or sRAGE had a markedly decreased area on days 1, 3, 7 and 14 after implantation (Fig. 3a).

Diminished tumour size in the setting of RAGE blockade indicated that RAGE might modulate cellular proliferation and/or cell death. Compared with mock-transfectants, C6 glioma expressing full-length RAGE exhibited enhanced incorporation of 5-bromo-2′-deoxyuridine (BrdU) on days 1 and 3 after implantation (Fig. 3b). In contrast, tail-deletion RAGE and sRAGE transfectants exhibited a decrease in incorporation of BrdU on days 1, 3 and 7 compared with mock-transfected clones (Fig. 3b). However, rates of apoptosis were low (0.2–0.5% from days 1–14) and did not change among the various transfectants. Similar results were obtained when sRAGE was administered intraperitoneally; on days 1, 3 and 7 after injection, decreased proliferation was noted in the presence of sRAGE (Fig. 3c) and the apoptotic rate was not different from that observed in mice receiving murine serum albumin (MSA) (data not shown). Consistent with the versatility of tumour cells was their ability to escape the suppression of cell growth associated with blockade of RAGE subsequent to day 14. This is when most tail-deletion and sRAGE transfectants, and tumour cells subjected to parenteral administration of sRAGE were first measurable. Subsequently, tumours from all groups escaped latency induced by blockade of RAGE and displayed equivalent growth rates (data not shown).

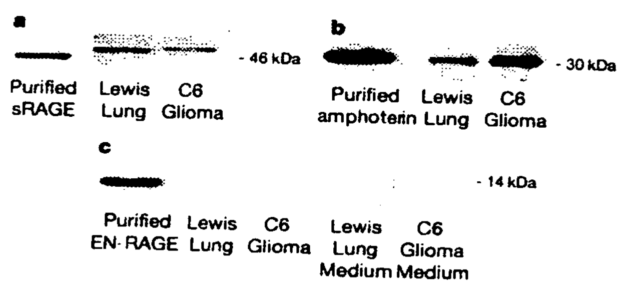

Figure 1 RAGE and amphoterin are expressed in tumour cells. **a–c**, Cell lysates or supernatant (medium) were prepared from cultured tumour cells and subjected to immunoblotting for RAGE (**a**), amphoterin (**b**), or EN-RAGE (**c**).

Amphoterin may provide a surface for assembly of fibrinolytic complexes leading to generation of plasmin, a central molecule in activation of matrix metalloproteinases (MMP)[14,15], indicating a mechanism by which RAGE–amphoterin might enhance invasive properties of tumour cells. On day 21 after implantation, those tumours overexpressing full-length RAGE had increased activity of MMP-9 and MMP-2 compared with mock-transfected C6 glioma (Fig. 3d). Tumours raised from tail-deletion RAGE or sRAGE in transfected clones, however, showed diminished MMP-9 and MMP-2 activity compared with those from mock-transfected clones (Fig. 3d).

Consistent with these *in vivo* observations, tail-deletion RAGE and sRAGE transfectants plated on amphoterin-coated matrices had diminished proliferation compared with mock-transfected C6 glioma (Fig. 4a). Further, overexpression of full-length RAGE in C6 glioma grown on amphoterin enhanced proliferation (Fig. 4a). These findings were selective for growth on amphoterin, as proliferation did not vary between mock-, full-length RAGE-, tail-deletion RAGE- and sRAGE-transfected C6 glioma grown on substrates with adsorbed bovine serum albumin (data not shown). Similar inhibition of cellular proliferation was observed when sRAGE was added to wild-type C6 glioma on amphoterin-coated matrices (data not shown).

As our findings indicated that RAGE and amphoterin mediated invasion and migration of implanted C6 glioma, we assessed these properties *in vitro*. Compared with mock-transfected C6 glioma, those cells with full-length RAGE demonstrated enhanced invasion through Matrigel (Fig. 4b). In contrast, transfected clones bearing the tail-deletion RAGE mutant or overexpressing sRAGE had diminished invasion (Fig. 4b). In the presence of sRAGE, anti-RAGE F(ab′)$_2$ or anti-amphoterin F(ab′)$_2$, significant suppression of invasion was observed (Fig. 4b). Similarly, blockade of RAGE–amphoterin suppressed migration of C6 glioma *in vitro* (data not shown). Also, mock- and full-length RAGE transfected C6 glioma

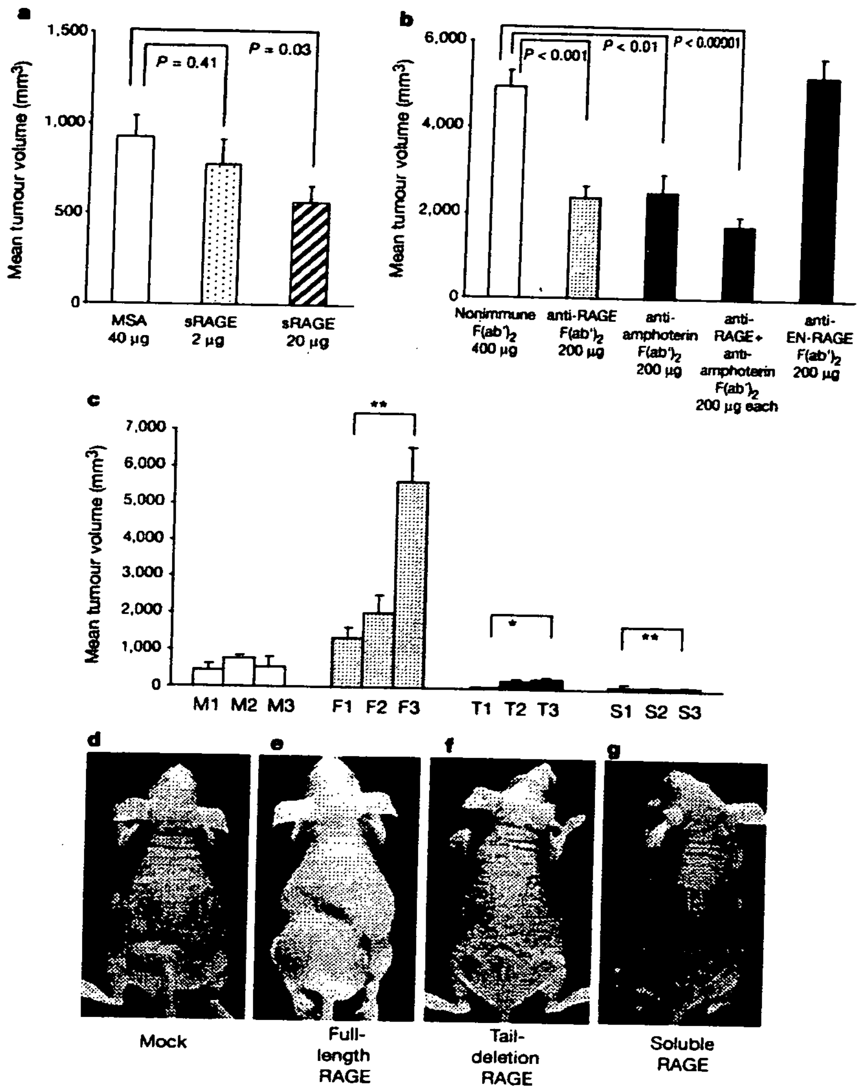

Figure 2 RAGE–amphoterin blockade suppresses growth of implanted C6 glioma. **a**, C6 glioma cells were implanted into immunocompromised mice and mean tumour volume on day 21 is shown, $n = 10$ per group. MSA, murine serum albumin; sRAGE, soluble RAGE. **b**, Mice with severe combined immunodeficiency (SCID) were injected with C6 glioma cells and the indicated F(ab′)$_2$ fragments. Mean tumour volume on day 21 is shown; $n = 7$ per group. **c**, Mock or RAGE/RAGE mutant C6 glioma cells were injected into immunocompromised mice and mean tumour volume on day 21 is shown. Asterisk, $P < 0.01$, double asterisk $P < 0.001$ versus mock-transfected clones. **d–g**, Representative photographs on day 21 of mice bearing the indicated transfected clone are shown. Scale bar, 1 cm.

spread readily on amphoterin-coated matrix (Fig. 4c, d), tail-deletion RAGE and sRAGE-transfected cells (Fig. 4e, f) had a marked reduction in their ability to extend processes necessary to migrate into the surrounding matrix.

To examine the molecular mechanisms underlying RAGE–amphoterin-mediated effects on tumour properties, we focused on the MAP kinase family of signalling effector molecules, as these mediators are involved in cellular proliferation, invasion and activation of MMPs[16–23]. Although activation of p44/p42, p38 and SAP/JNK MAP kinases was enhanced in full-length RAGE transfectants plated on amphoterin, a marked reduction in activation of these kinases on amphoterin was noted in tail-deletion RAGE and sRAGE transfectants (Fig. 4g–i, left). In contrast, activation of MAP kinases did not differ among the transfectants grown on BSA, suggesting the specificity of amphoterin–RAGE interaction in activating these key cell signalling molecules (Fig. 4g–i, right).

Another potent mechanism by which growth and spread of tumour cells may be modulated is by suppression of neovascularization. To explore whether RAGE blockade impaired this process, basic fibroblast growth factor (b-FGF)-laden pellets were placed into a corneal pocket, and new capillary growth from the corneal limbus was observed[23]. New vessel growth was not different in C57BL/6J mice treated with either MSA or sRAGE for five days ($3.1 \pm 0.3\,mm^2$ and $2.7 \pm 0.2\,mm^2$ angiogenic area, respectively; $P > 0.05$).

As the ability of tumour cells to proliferate and invade beyond homeostatic boundaries is a central means by which the host succumbs to tumour, it was important to ascertain whether RAGE might contribute to metastases. We used the Lewis lung carcinoma model, in which distant metastases flourish upon removal of the primary tumour. Both RAGE and amphoterin were present in Lewis lung carcinoma cells (Fig. 1a, b), and could be localized to the cell surface by immunocytochemistry (not shown). We first prepared stably transfected Lewis lung carcinoma cells overexpressing sRAGE. However, following implantation into C57BL/6J mice, marked suppression of local tumour growth resulted (data not shown), thereby abrogating the usefulness of this model. As an alternative strategy, sRAGE was administered just before and after resection of primary tumours resulting from inoculation with wild-type Lewis lung carcinoma cells. Compared with MSA, animals receiving sRAGE at 100 µg per day demonstrated a marked decrease in the number of lung surface metastases (8.7 ± 1.4 and 1.0 ± 0.3, respectively; $P < 0.0001$) and metastatic burden as judged by lung weight (385.6 ± 39.8 mg and 188.7 ± 6.7 mg, respectively; $P < 0.001$). Lung surface metastases observed in MSA-treated mice were virtually undetectable in mice treated with sRAGE (see Fig. 3 in Supplementary Information).

To study early RAGE-mediated events in metastasis, fluorescently labelled Lewis lung carcinoma cells were intravenously injected into mice; 24 h later, cell number in the lungs was assessed. Compared with MSA-treated mice, a significant reduction in tumour cell number under high-powered field was noted in the presence of sRAGE (1.1 ± 0.03 and 0.4 ± 0.02, respectively; $P < 0.0001$). On day 14, the number of lung surface metastases was reduced in mice treated with sRAGE compared with those receiving MSA ($13.0, \pm 1.5$ and 47.6 ± 4, respectively; $P < 0.00001$), as was lung weight (194.8 ± 8.8 mg and 405.8 ± 25.3 mg, respectively; $P < 0.00001$).

The critical test of our hypotheses was whether blockade of RAGE–amphoterin might affect endogenous growth of tumours. We tested these concepts in spontaneously appearing papillomas in mice overexpressing v-Ha-*ras* transgene. After stimulation of the skin with a tumour promoter such as phorbol 12-myristate 13-acetate (PMA), papillomas appear in >90% of mice within six weeks[24].

RAGE and amphoterin were highly expressed in endogenously formed papillomas in PMA-treated transgenic mice (Fig. 5b, c). To

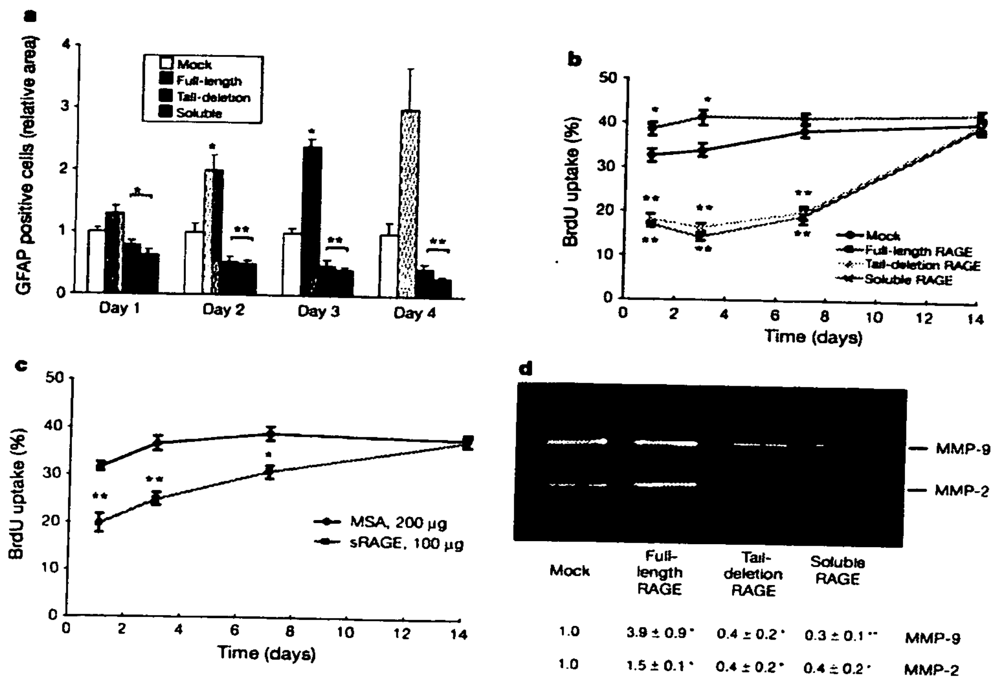

Figure 3 Blockade of RAGE suppresses tumour proliferation and expression of MMPs. **a**, Mean area at the centre of tumours raised from the indicated clones of C6 glioma was determined by immunohistochemical determination of GFAP-expressing tumour cells; $n = 5$ mice. **b,c**, The indicated C6 glioma transfectants (**b**) or C6 glioma in the presence of MSA or sRAGE (**c**) were implanted into mice; 1 h before sacrifice, mice were injected with BrdU; tissue was retrieved and subjected to immunohistochemistry to assess incorporation of BrdU. **d**, Tumours ($n = 3$ per clone) were retrieved 21 days after implantation and zymographic determination of MMP9 or MMP2 activity performed. Results of densitometric analysis are indicated; 1.0 is arbitrarily assigned to density of bands in mock-transfected tumours. In **a-d**, asterisk, $P < 0.05$, double asterisk, $P < 0.01$ versus mock or MSA.

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study RAGE–amphoterin blockade, sRAGE or MSA was administered to transgenic mice. After six weeks, marked suppression of papilloma formation was noted in sRAGE-treated transgenic animals compared with treatment with MSA (Fig. 5e, f; mean papillomas per animal, 3.8 ± 1.0 and 0.3 ± 0.3, respectively; $P < 0.01$).

Consistent with earlier observations, incorporation of BrdU was attenuated in papillomas studied from sRAGE-treated transgenic mice compared with those retrieved from mice receiving MSA (22 ± 2% and 39 ± 2%, respectively; $P < 0.0001$), and no differences in apoptotic rates were observed between papillomas in sRAGE-treated and MSA-treated mice (0.1 ± 0.1% and 0.2 ± 0.1%, respectively; $P > 0.05$).

In embryonic development, amphoterin and RAGE co-localize at the leading edge of advancing neurites, indicating a role in neuronal

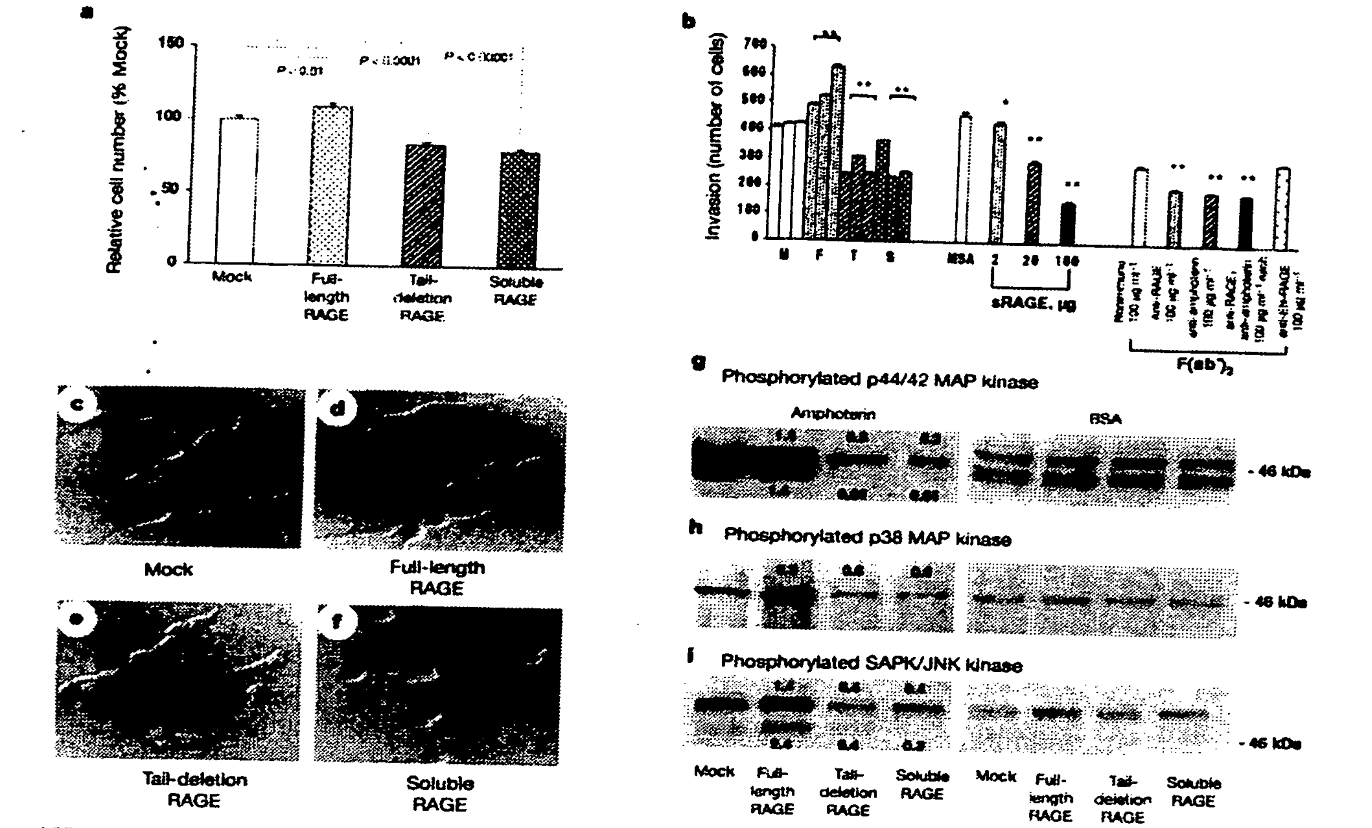

Figure 4 Effects of RAGE–amphoterin blockade on C6 glioma: *in vitro* analyses. **a**, The indicated clones of C6 glioma were grown on matrix coated with amphoterin. Cell number was quantified 72 h later and results reported as relative cell number, compared with that observed in mock-transfectants. **b**, The number of cells invading Matrigel is shown. Asterisk, $P < 0.05$; double asterisk $P < 0.00001$ versus respective control. **c–f**, Appearance of the indicated mock- or RAGE/RAGE C6 glioma mutants was assessed on amphoterin. Scale bar, 20 µm. **g–i**, The indicated clones of C6 glioma were plated on amphoterin (left) or BSA (right) for 90 mins; cells were retrieved and immunoblotting performed to assess activation of p44/p42 (**g**), p38 (**h**) and SAP/JNK MAP kinases (**i**). Densitometric analysis for kinase activation on amphoterin compared with mock-transfected clones (1.0) is indicated.

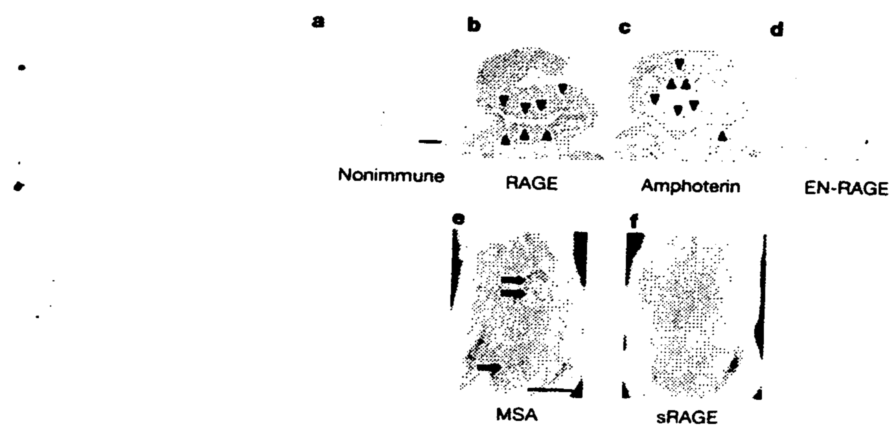

Figure 5 Blockade of RAGE suppresses endogenous growth of papillomas. **a–d**, After six weeks of local application of PMA, papillomas forming in transgenic mice overexpressing v-Ha-*ras* were examined by immunohistochemistry using the indicated IgG. Arrowheads indicate the sites of most intense immunostaining for RAGE and amphoterin; these areas co-localized with sites of highest levels of BrdU incorporation (not shown). Scale bar, 100 µm. **e**,**f**, After six weeks, increased numbers of papillomas (arrows) were observed in mice receiving MSA (**e**: $n = 8$) versus sRAGE (**f**; $n = 7$). Scale bar, 1 cm.

development[3,15]. Here we extend these concepts to tumour growth and metastasis, and demonstrate RAGE–amphoterin involvement in cellular proliferation and invasiveness and that it is also central for tumour growth and spread. Blockade of RAGE–amphoterin in the tumour bed forces cells into a period of dormancy[27], characterized by diminished proliferation, invasion and MMP activity; properties linked to the MAP kinase family of signal transduction effector molecules. We have developed a new model in tumour biology and identified RAGE as a target for therapeutic strategies to suppress local tumour growth and distant metastases. These new therapies will probably enhance the benefit of other anti-tumour strategies, such as those designed to diminish neovascularization, proliferation and evasion of the host immune response. □

Methods

Immunoblots

Rat C6 glioma and murine Lewis lung carcinoma cell lines were purchased from the American Type Culture Corporation (ATCC). Immunoblotting was performed with 30 μg cell extract[4] or cellular supernatant and using rabbit anti-RAGE IgG[3], rabbit anti-rat amphoterin IgG[3], rabbit anti-EN-RAGE IgG[8] or equal amounts of nonimmune IgG (25 μg ml^{-1}). In all cases, control recombinant proteins (0.5 μg) were murine soluble RAGE, rat amphoterin and bovine EN-RAGE. All control proteins crossreact with murine and rat antigens.

Confocal microscopy

C6 glioma were fixed with paraformaldehyde (2%) and confocal microscopy (Zeiss) was performed using anti-RAGE and anti-amphoterin IgG. Secondary antibodies included FITC-conjugate (for amphoterin) and TRITC-conjugate (for RAGE) (Sigma; 1:200 dilution in both cases).

Immunohistochemistry

Implanted C6 glioma were excised and fixed with formalin: paraffin-embedded sections (5 μm thick) were prepared from the exact centre of the tumours, and subjected to immunohistochemistry with anti-glial fibrillary acidic protein Ig (Sigma). Microscopic images (Zeiss) of GFAP-stained sections were scanned into a computer and image analysis for determination of area using software provided by MediaCybernetics[5]. For endogenously forming papillomas, sections were prepared as above and subjected to immunohistochemistry using rabbit nonimmune IgG, anti-RAGE IgG, anti-amphoterin IgG or anti-EN-RAGE IgG (2 μg ml^{-1} each).

Stably transfected C6 glioma complemetary DNAs for human full-length, cytosolic tail deletion and soluble RAGE[2] were inserted into the pcDNA3 vector (Invitrogen). C6 rat glioma cells were transfected by using Lipofectamine (Life Technologies). Cells were selected in the presence of geneticin (G418), 1.5 mg ml^{-1} (Life Technologies), and individual clones were isolated by limiting dilution. Mock-transfectants contained vector alone.

Local tumour growth

Rat C6 glioma cells (1×10^5 in 0.1 ml PBS) were injected into the dorsal midline of female NCR immunocompromised mice, aged 4–6 weeks (Taconic Farms, Germantown, NY). Alternatively, rat C6 glioma cells were injected into the dorsal midline of female mice with severe combined immunodeficiency (SCID; Taconic Farms). Tumours were measured with calipers and the volume was calculated: $V = \pi \times h(h^2 + 3a^2)/6$, where h = height of the tumour segment; a = (length + width of the tumour)/4; and V = volume of the tumour. In all cases, viability of injected cells was > 95% by exclusion of trypan blue upon injection into mice. In certain mice, one hour before sacrifice, 1 mg BrdU (Sigma) was injected by intraperitoneal administration. Tumour tissue was retrieved, fixed in formalin (10%) and paraffin-embedded sections were prepared. Sections were stained with haematoxylin and eosin, or were simultaneously immunostained for detection of BrdU (anti-BrdU antibody; Accurate, Westbury, NY) and for *in situ* apoptosis detection (Trevigen, Gaithersburg, MD).

Tumour metastases

Lewis lung murine carcinoma cells (2×10^6 in 0.1 ml PBS) were injected into the dorsal midline of male, 6–8-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Primary tumours were surgically excised when tumour volume was 1,500 mm^3 (day 14). For three days before sacrifice, mice received sRAGE or MSA once daily, 21 days after removal of primary tumour. Weight of the lungs and numbers of lung surface metastases were determined under ×4 magnification using an Olympus microscope after intratracheal injection of India Ink (15%). In other experiments, Lewis lung carcinoma cells were labelled with Vybrant CFDA (Molecular Probes, Eugene, OR). 2×10^5 cells in 0.1 ml PBS were injected intravenously into C57BL/6J mice and animals were sacrificed 24 h or 14 days after injection. Lungs were removed, fixed and paraffin-embedded sections prepared (5 μm thick). Fluorescence microscopy was used to identify tumour cells; 60 sections were assessed per mouse.

Endogenous tumour formation

Male transgenic mice (hemizygous) carrying the activated v-Ha-*ras* transgene[29] were purchased from Taconic Farms. At age 8 weeks, mice were housed in single cages and their backs shaved (4 × 2 cm). PMA (Sigma), 5 μg dissolved in acetone (200 μl), was administered locally to the shaved area twice weekly for 6 weeks.

Tumour properties

To assess proliferation, the indicated C6 glioma cells (1×10^3 cells per well) were incubated on culture wells coated with amphoterin (10 μg ml^{-1}) or BSA (20 μg ml^{-1}). MSA or sRAGE was added, and three days later cell number was assessed using the CyQUANT Cell Proliferation Assay kit (Molecular Probes). Absence of cell death was documented by exclusion of trypan blue. Invasion and migration assays were performed as described[28,29].

For the assessment of MMP-2 and 9 activity[30], tumour tissue was retrieved on day 21. Equal amounts of protein were subjected to electrophoresis on gelatin-laden gels (0.1%) (Novex) and results normalized to the weight of the tumour.

To examine the properties of the tumours grown on amphoterin, C6 glioma cells, 3×10^4 cells per well, were added to plastic dishes (Nunc, Naperville, IL) coated with purified amphoterin (10 μg ml^{-1}) or BSA (20 μg ml^{-1}). After 18 h, cells were photographed under phase contrast microscopy. In other studies, cells were retrieved 90 min after plating, and extracts (30 μg protein) subjected to electrophoresis and immunoblotting to detect phospho-p44/42 MAP kinase (Thr202/Tyr204), phospho-p38 MAP kinase (Tyr180/Tyr182), or phospho-SAPK/JNK (Thr183/Tyr185) (New England BioLabs, Beverly, MA).

Data analysis

In all experiments, mean ± standard error is reported. Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA); where indicated, individual comparisons were performed using Students' *t*-test.

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Neurotoxicity induces cleavage of p35 to p25 by calpain

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Cyclin-dependent kinase 5 (cdk5) and its neuron-specific activator p35 are required for neurite outgrowth and cortical lamination[1–3]. Proteolytic cleavage of p35 produces p25, which accumulates in the brains of patients with Alzheimer's disease[4]. Conversion of p35 to p25 causes prolonged activation and mislocalization of cdk5. Consequently, the p25/cdk5 kinase hyperphosphorylates tau, disrupts the cytoskeleton and promotes the death (apoptosis) of primary neurons. Here we describe the mechanism of conversion of p35 to p25. In cultured primary cortical neurons, excitotoxins, hypoxic stress and calcium influx induce the production of p25. In fresh brain lysates, addition of calcium can stimulate cleavage of p35 to p25. Specific inhibitors of calpain, a calcium-dependent cysteine protease, effectively inhibit the calcium-induced cleavage of p35. *In vitro*, calpain directly cleaves p35 to release a fragment with relative molecular mass 25,000. The sequence of the calpain cleavage product corresponds precisely to that of p25. Application of the amyloid β-peptide Aβ(1–42) induces the conversion of p35 to p25 in primary cortical neurons. Furthermore, inhibition of cdk5 or calpain activity reduces cell death in Aβ-treated cortical neurons. These observations indicate that cleavage of p35 to p25 by calpain may be involved in the pathogenesis of Alzheimer's disease.

The open reading frame of p35 does not contain introns[2], so alternative splicing cannot account for the generation of p25. Internal initiation of translation of p35 messenger RNA is also unlikely to produce p25 because there is no internal methionine near the beginning of the p25 sequence. Proteolytic cleavage is, therefore, the most likely mechanism for conversion of p35 to p25 (Fig. 1a).

Despite extensive efforts to identify p25 in the mouse, only full-length p35 was detectable during embryonic development and in the adult (data not shown). We next sought to determine whether p25 could be produced *in vivo* under certain experimental conditions. We found that 4 h of focal ischaemia, induced by middle cerebral artery occlusion in mice, produced p25 in the ipsilateral cortex but not in the control contralateral cortex (Fig. 1b). The conversion of p35 to p25 caused it to relocalize to the cytoplasm (Fig. 1c), as reported previously[4].

To investigate the mechanism of the conversion of p35 to p25 further, we tested for conditions that would induce the appearance of p25 in cultured primary cortical neurons. Treatment with hydrogen peroxide stimulated cleavage of p35 to p25 in primary neurons (Fig. 1d). Other insults such as treatment with the excitatory amino-acid glutamate also caused the production of p25 in cortical neurons at high concentrations of glutamate (Fig. 1e). An increase in intracellular calcium levels, caused by the calcium ionophore ionomycin, stimulated efficient conversion of p35 to p25 (Fig. 1f). These results indicate that neurotoxicity induces cleavage of p35 to p25 and suggest a role for calcium in this process.

To identify the protease that cleaves p35 to p25, we sought to recapitulate the proteolytic cleavage event. In fresh mouse brain lysates, 1 mM Ca^{2+} efficiently stimulates the cleavage of p35 (Fig. 2a). The cleavage product is likely to be p25, as it has a relative molecular mass of 25K and co-migrates with recombinant p25 expressed in COS-7 cells (lane 1). Also, like p25, it is specifically recognized by the p35 carboxy-terminal-specific antibody, but not by the p35 amino-terminal-specific antibody (see Supplementary Information).

p35 contains no obvious consensus sequences for cleavage by known proteases. To identify the protease activated by calcium, we tested protease inhibitors with different specificities for their effectiveness in inhibiting the calcium-stimulated p35 conversion. Calpeptin and calpain inhibitor II, which inhibit the calcium-dependent cysteine protease calpain, completely inhibited p35 cleavage (Fig. 2b, lanes 3–4), whereas the general cysteine protease inhibitor leupeptin partially inhibited p35 cleavage (lane 8). A titration of four calpain-specific inhibitors shows that 10 nM calpeptin, 100 nM calpain inhibitor I, 100 nM calpain inhibitor II and 5 nM calpastatin effectively inhibit p35 conversion (Fig. 2d and Supplementary Information), consistent with the reported median inhibitory concentration (IC_{50}) values for these inhibitors[5]. The lack of effect of the cdk5 inhibitor roscovitine indicates that cdk5 activity may not be necessary for cleavage to occur (Fig. 2b, lane 9).

M-calpain and μ-calpain are the two main isoforms of calpain in the brain[6]. The two calpains differ in their calcium requirements but have similar substrate specificities. μ-calpain requires 3–50 μM calcium for half-maximal activity, whereas m-calpain requires 0.2–1 mM calcium for activity. To determine whether calpain was indeed activated in the conditions tested *in vitro*, we examined the cleavage of a well characterized calpain substrate, non-erythroid α-spectrin (also known as α-fodrin)[5]. One millimolar calcium, which stimulated conversion of p35 to p25 in mouse brain lysates, also led to cleavage of endogenous spectrin into the characteristic 145K and 150K fragments, indicating that calpain was activated (Fig. 2c). Furthermore, spectrin cleavage was inhibited by calpeptin,

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Cancer: Checkpoint for invasion

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Both benign and malignant tumours grow in an uncontrolled way. But it is only cells of malignant tumours that invade surrounding tissues and travel to distant organs (metastasize). Conventional wisdom used to hold that invasion and metastasis are late events — often 'too late' — in the clinical course of a patient's cancer. However, we now know that invasion can be both early and clinically 'silent'. An understanding of the molecular basis for this aggressiveness could lead to therapies that block the transition of a tumour from benign to malignant, and keep local disease in check. Taguchi and colleagues[1], writing on page 354 of this issue, have now identified proteins called RAGE and amphoterin as a receptor–ligand pair in a molecular checkpoint that regulates not only the invasiveness but also the growth and movement of tumour cells — the trio of characteristics required for malignancy.

The threat of tumour invasiveness is exemplified by the fact that brain cancer does not need to metastasize to kill a patient. The growth of a brain tumour mass in the confined area of the skull causes compression damage; in addition, local invasion by brain tumour cells can destroy surrounding, normal brain tissue. In many cases, brain tumour cells can move away from the primary tumour to reach other sites within the brain. Such insidious invasive behaviour may represent the inappropriate use of a programme responsible for the outgrowth of neuronal protrusions called neurites during normal neuronal development. Indeed, cancer invasion in general may be a deregulated form of a physiological invasion process required for neuronal wiring in the embryo, tissue remodelling, the formation of blood vessels, and healing[2].

Amphoterin is a key protein in normal neurite outgrowth. It is a heparin-binding protein that is abundant in extracellular regions of the developing brain and other organs. Antibodies that recognize amphoterin block neurite outgrowth under experimental conditions; so, interactions of amphoterin with neuronal surfaces appear to be required for the extension of neuronal processes. Amphoterin's receptor on the cell surface is a protein called RAGE (for 'receptor for advanced glycation end products')[3]. RAGE is a receptor for many different ligands, and is a member of the immunoglobulin superfamily of cell-surface molecules. It gains its name, and was first identified, because it recognizes potentially damaged, glycated proteins (that is, those with carbohydrate polymers attached to them) that accumulate during diabetes. Amphoterin and RAGE localize together at the leading edge of advancing neurites during embryonic development[3]. Taguchi *et al.*[1] recognized the implications of this result for pathological processes such as cancer invasion.

The complete set of characteristics of a malignant tumour is induced by a variety of cellular programmes and pathways, many of which are not yet fully defined. Faced with this complexity, the best way to link a molecule causally to malignancy is to start with a cell that is already malignant, and to attempt to block the molecule or pathway of interest. This was the tack taken by Taguchi *et al.*[1], who used several approaches to block the RAGE–amphoterin axis in C6 glioma brain tumour cells. Inhibitory strategies included administering the soluble form of the ligand-binding domain of RAGE, of anti-RAGE antibodies, or of anti-amphoterin antibodies, or introducing defective forms of RAGE into C6 glioma cells. *In vitro* and in animal models of cancer, all of these treatments significantly inhibited the growth, motility and local

invasion of tumour cells, as well as metastasis of the cells to the lungs. The treatments even inhibited the spontaneous growth of papillomas (benign skin cancers) in mice overexpressing the v-Ha-*ras* oncogene. How might the RAGE–amphoterin pathway have these effects?

Let's take a look first at invasion. Regulation of the molecular events necessary for invasion — whether physiological or malignant — involves spatial and temporal coordination, as well as cyclic on–off processes, at the level of individual cells (Fig. 1). Motility, coupled with regulated, intermittent adhesion to the extracellular matrix and degradation of matrix molecules, allows an invading cell to move through the three-dimensional matrix. At the leading edge of the motile cell, receptor–ligand and proteolysis–antiproteolysis complexes coordinate sensing, protrusion, burrowing and traction of the cell[4, 5].

Figure 1 Spatial and temporal regulation of cellular invasion of the extracellular matrix. Full legend

High resolution image and legend (34k)

It was already known that amphoterin at the cell surface can act as a nucleating site for generation of the protein-degrading complex plasmin[6]. This complex can activate matrix metalloproteinases (MMPs), which are enzymes that degrade extracellular matrix molecules[5]. Taguchi *et al.*[1] now report that blocking RAGE results in decreased activity of MMP-2 and MMP-9 — molecules previously associated with invasion of both cancer cells and neurites. Localized proteolysis of matrix molecules may loosen up, or open up, the dense meshwork of matrix molecules being invaded. Proteolysis at the migration front may also liberate previously bound growth factors or motility-stimulating molecules.

The RAGE–amphoterin complex also suppresses tumour growth, but how does it coordinate these three inhibitory effects — on growth, on motility and on invasion? Proteins called cytokines, as well as proteins found in the extracellular matrix, trigger signalling cascades that regulate both cell migration and proliferation. Bifurcation of this signalling pathway occurs at the level of mitogen-activated protein kinase (MAPK) signalling modules. Three coexisting modules — $p38^{MAPK}$, JNK and $p42/p44^{MAPK}$ — exchange signals between the cell surface, the cytoskeleton and the nucleus.

When a ligand stimulates the cell through that ligand's receptor, some or all of these MAPK modules can be activated, directly or indirectly, as can the small GTP-hydrolysing proteins (GTPases) Ras, Cdc42, Rac and Rho[7, 8]. Activated MAPK modules propagate signals downstream into the nucleus to activate genes encoding growth inducers, MMPs and adhesion receptors. In parallel, these modules elicit further events that modify the myosin and actin filaments of the cytoskeleton. So all three MAPK modules can act as relay stations for the regulation of growth, motility and invasion. Taguchi *et al.*[1] show that RAGE–amphoterin acts simultaneously through all three MAPK modules, explaining how blocking RAGE will experimentally suppress all components of the malignant phenotype.

'Signal-transduction therapy' is a treatment strategy in which key, hyperactive cellular signalling pathways that cause disease are targeted. The trick is to find a rheostat in the cell's circuitry that is not bypassed by collateral or compensatory paths. The RAGE–amphoterin pathway may well fulfil these criteria.

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